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(54) Title: METHODS FOR THE TREATMENT OF NEURONAL ATROPHY-ASSOCIATED DEMENTIA

(57) Abstract

The invention features methods of diagnosis and treating a patient having Alzheimer's disease or other neuronal atrophy-associated dementia by determining or altering, respectively, the level of activity of pathways from the endoplasmic reticulum to lysosomes in the patient.

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METHODS FOR THE TREATMENT OF NEURONAL ATROPHY-ASSOCIATED DEMENTIA

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Background of the Invention

The invention relates to methods of identifying compounds for the treatment of Alzheimer's disease (AD) and non-AD neuronal atrophy-associated dementia.

Rare early-onset forms of familial Alzheimer's disease (FAD) are inherited as autosomal dominant diseases. Mutations in the broadly expressed transmembrane amyloid precursor protein (APP), although extremely rare, were the first FAD-causing genetic defects identified and are associated with abundant cerebrovascular β-amyloid, a major neuropathological feature of AD. All of these mutations appear to influence the proteolytic processing of APP, modifying the amount of and/or length of the Aβ peptide, the major component of β-amyloid. Mutations of the presenilin genes (PS1 and PS2) were more recently identified as causing more than half of all cases of early-onset FAD. Among other proposed effects, presenilin mutations influence the production of the 42 and 40 kDa forms of Aβ1(Aβ1-42 and Aβ1-40) by favoring the former. The existing animal models of AD, other than aged primates or dogs, make use of mutant APP and mutant presenilin to create mice that deposit β-amyloid.

An early hallmark of AD pathology is activation of the lysosomal system (LS) (Cataldo et al., Neuron 14:671-680, 1995; Nixon et al., Trends Neurosci. 18:489-496, 1995; Cataldo et al., Adv. Exp. Med. Biol. 389:271-280, 1996; Cataldo et al., J. Neurosci. 16:186-199, 1996). Despite concerted efforts,

there is an incomplete understanding of the mechanism of LS activation and its role in AD pathogenesis.

Thus, there is a need to define the pathogenic significance of the early and progressive activation of the LS in neuronal atrophy-associated dementias such as AD. There is also a need for assays for compounds that are useful for the treatment of neuronal atrophy-associated dementias, and particularly for compounds that reduce Aβ formation. Moreover, there is a need for better tools for the diagnosis of neuronal atrophy-associated dementias.

Summary of the Invention

In a first aspect, the invention features a method of diagnosing Alzheimer's disease or other neuronal atrophy-associated dementia in a human patient, the method including determining the level of activity of pathways from the endoplasmic reticulum to lysosomes in the patient, and comparing the level of the activity to normal levels, wherein an increase in the level of activity relative to normal indicates AD or other neuronal atrophy-associated dementia.

In one embodiment, the measuring includes measuring the level of activity of pathways from the endoplasmic reticulum to lysosomes in a cell of the patient. Preferably the cell is a neuron, a fibroblast, or an endothelial cell. In a related embodiment, the measuring includes measuring the level of activity of pathways from the endoplasmic reticulum to lysosomes in a biological fluid of the patient.

In a second aspect, the invention features a method for identifying a candidate compound as a compound that is useful for the treatment of AD or other neuronal atrophy-associated dementia. The method includes: a) providing a cell; b) contacting the cell with the candidate compound; and c) determining the activity of pathways from the endoplasmic reticulum to

lysosomes, wherein a decrease in the activity, relative to the activity in a control cell not contacted with the candidate compound, identifies the candidate compound as a compound that is useful for the treatment of AD or other neuronal atrophy-associated dementia.

In a third aspect, the invention features a method for identifying a candidate compound as a compound that is useful for the treatment of AD or other neuronal atrophy-associated dementia. The method includes: a) providing a cell; b) contacting the cell with a candidate compound that decreases the activity of pathways from the endoplasmic reticulum to lysosomes; and c) determining the ability of the cell to withstand cytotoxic challenge such as, but not limited to, oxidative stress, $A\beta_{,,}$ hypoxia, or metabolic challenge, wherein an increase in cell survival in a cell contacted with the compound, relative to survival of a cell not contacted with the compound, identifies the compound as one that is useful for the treatment of AD or other neuronal atrophy-associated dementia.

In a fourth aspect, the invention features another method for identifying a candidate compound as a compound that is useful for the treatment of AD or other neuronal atrophy-associated dementia. The method includes: a) providing a cell; b) contacting the cell with a candidate compound that decreases the activity of pathways from the endoplasmic reticulum to lysosomes; and c) determining the levels of $A\beta$ produced by the cell, wherein a decrease produced by a cell contacted with the compound compared to a control cell not contacted with the candidate compound identifies the candidate compound as a compound that is useful for the treatment of AD or other neuronal atrophy-associated dementia.

In preferred embodiments of the second, third, or fourth aspect, the cell is in a human or a mouse, or is from a human or a mouse. The cell may contain a polypeptide sequence including a mutation that is present in a human

with familial AD. In various preferred embodiments, the polypeptide sequence includes PS1 or PS2, and the mutation is P117L, M146L, M146V, S169L, M233T, or A246G, or the polypeptide includes APP. The cell can be *in vitro*. The cell can be, for example, a fibroblast, an endothelial cell, or a neuron.

The cell can be in an animal, such as a mouse, rat, dog, cat, or monkey, or the cell can be in culture. The cell can be, for example, a fibroblast, an endothelial cell, or a neuron. In one embodiment, the autophagy inhibitor is leupeptin.

In a fifth aspect, the invention features a method for treating a patient with AD or other neuronal atrophy-associated dementia, the method including administering to the patient a compound that decreases the activity of pathways from the endoplasmic reticulum to lysosomes.

In various embodiments, the patient has sporadic AD, familial AD, Down's syndrome (DS), Parkinson's disease, or has a mutation in their PS1 gene, their PS2 gene, or their APP gene.

Preferred compounds are 3-methyladenine (3MA), a derivative of 3MA, leucine, histidine, and vinblastine. Preferably, the compound further reduces Aβ formation.

In all of the foregoing aspects of the invention, the preferred neuronal atrophy-associated dementia is AD.

By "pathways from the endoplasmic reticulum to lysosomes" is meant the autophagy pathway and the pathway for the direct conversion of ER to lysosome. Activity of these pathways can be measured using methods described herein. A decrease or a reduction in the activity of pathways from the endoplasmic reticulum to lysosomes is any diminishment of the activity relative to a control cell. Preferably, the decrease in activity is at least 5%, more preferably 10%, and most preferably 25% or even 50%. The percent change is usually measured for a period of hours or days, but can be measure in

terms of weeks or even longer.

By "dementia" is meant deterioration of intellectual faculties, such as memory, concentration, and judgment, resulting from an organic disease or a disorder of the brain. By "neuronal atrophy-associated dementia" is meant dementia caused by a loss in neurons and includes dementia associated with AD, Parkinson's disease, frontotemporal dementia, DS, and amyolateral sclerosis (ALS).

By "metabolic challenge" is meant exposure of a cell to conditions which are generally toxic or harmful to the cell, including disruption of calcium homeostasis, trophic factor deprivation, glucose or amino acid starvation, hypoxia, excitotoxic challenge, and disruption of a cellular proteolytic system such as the lysosomal system.

The invention provides methods for identifying drugs useful for the treatment or prevention of AD or other neuronal atrophy-associated dementia. Additionally, the invention provides new drug targets for rational drug design. Also provided by the invention are compounds that may be useful for the treatment or prevention of AD or other neuronal atrophy-associated dementia.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Brief Description of the Drawings

Figs. 1A-1D are a series of photographs showing abnormally large rab5-immunopositive early endosomes in pyramidal neurons from individuals with sporadic AD (arrow in Fig. 1B) when compared to controls (Fig. 1A). Enlarged early endosomes were not seen in individuals with PS mutations (Fig. 1C) despite high levels of β-amyloid deposition (Fig. 1D, arrowheads).

Fig. 1E is a graph showing that the average endosomal volume per

neuron in the PS-FAD cases was similar to that of normal controls (control mean, 1.88%; PS-FAD mean, 2.20%; SAD mean, 5.04%).

Figs. 2A-2C are a series of photographs showing cathepsin D immunoreactivity. Similar to the SAD brain (Fig. 2B), PS-FAD brain (Fig. 2C) displayed denser cathepsin D immunoreactivity in pyramidal neurons in cortical laminae III and V compared to controls (Fig. 2A). In contrast to SAD, cathepsin D immunolabeling in PS-FAD was also increased in neurons of laminae II and IV of the prefrontal cortex. Senile plaques (arrowheads) displayed Cat D immunoreactivity.

Figs. 3A-3D are a series of photographs showing lysosomal system upregulation, as revealed by LAP enzyme cytochemistry, in $PS1_{M146L}$ mice (Fig. 3B) and $PS1_{M146L}$/APPswe transgenic mice (Figs. 3C and 3D) when compare d to a non-transgenic mouse (Fig. 3A).

Figs. 3E- 3H are photographs of LAP enzyme cytochemistry in control mice (Fig. 3F), $PS1_{M146L}$/APPswe transgenic mice (Figs. 3E and 3H), and $PS1_{M146L}$ mice (Fig. 3G) at the electron microscope level, again showing upregulution of the lysosomal system in the transgenic mice.

Figs. 4A-4C are a series of photographs showing the association of a biotinylated sMPR probe with Aβ-containing plaques in $PS1_{M146L}$ APPswe transgenic mice.

Figs. 5A and 5B are photographs of autophagic vacuoles (arrows) in control mice (Fig. 5A)and $PS1_{M146L}$/APPswe transgenic mice (Fig. 5B).

Fig. 5C is a photograph showing autophagic vacuoles are a subset of the population containing enzyme cytochemical reaction product for acid phosphatase activity.

Figs. 6A-6C are a series of photographs showing monodansyl-cadaverine labeling of autophagic vacuoles in control L cells (Fig. 6A) and $PS1_{P117L}$ transfected L cells (Figs. 6B and 6C).

Figs. 7A-7C are a series of photomicrographs showing increased numbers of autophagic vacuoles in N2a cells expressing PS1Δ9/APPswe (arrows Fig. 7C) when compared to untransfected N2a cells (Fig. 7A) and PS1wt/APPswe expressing N2a cells (Fig. 7B).

Fig. 8 is a schematic illustration showing quantitation of authophagic vaculoes in N2a cells overexpressing wild-type PS1, in untransfected N2a cells, and in PS1Δ9 expressing N2a cells.

Figs. 9A and 9B are photographs of hydrolase-positive lysosomes in control skin fibroblasts (Fig. 9A) and fibroblasts from an individual with a PS1 mutation (Fig. 9B).

Figs. 9C and 9D are photographs of protein disulfide isomerase in control skin fibroblasts (Fig. 9C) and fibroblasts from an individual with a PS1 mutation (Fig. 9D).

Fig. 9E is a photograph of LAMP in fibroblasts from an individual with a PS1 mutation.

Fig. 10A is a photograph of an autoradiograph showing Aβ generation is decreased in neuroblastoma cells following treatment with 3MA and leucine/histidine.

Fig. 10B is a schematic illustration showing by ELISA decreased Aβ secretion into the growth media following treatment of N2a cells with 3MA.

Fig. 10C is a photograph of a Western blot showing increased tau protein levels in 3MA-treated and vinblastine-treated PS1Δ9/APPswe N2a cells.

Fig. 11 is a schematic illustration showing a reduction in Aβ secretion into the media following 3MA treatment of primary cortical neurons isolated from normal mice.

Detailed Description of the Invention

We have discovered that two non-endosomal routes to the lysosome play a significant role in LS activation in AD, particularly in PS-FAD. This discovery allows for (i) the identification of compounds that are useful for the treatment of AD by assessing their ability to decrease LS activity originating from the endoplasmic reticulum to lysosomes; (ii) the diagnosis of AD based on assays that measure activity of pathways from the endoplasmic reticulum to lysosomes; and (iii) treatment of AD with drugs that decrease the activity of pathways from the endoplasmic reticulum to lysosomes (e.g., 3MA, leucine, histidine, or vinblastine).

One of the two routes is autophagy, the process by which cells digest their own cytoplasm to provide materials for new synthesis (Seglen et al., Semin. Cell Biol. 1:441-448, 1990). Based on studies in non-neural tissues, autophagy begins with a rate-limiting sequestration of cytoplasm in a membranous organelle (phagophore) of unknown origin and composition, but believed to be derived from ER membranes. The vacuole eventually formed (autophagosome) may fuse with a prelysosomal compartment, likely to be the late endosome. Autophagy plays a critical role in modulating cellular protein economy and remodeling cell architecture in response to physiological and pathological stimuli (Brunk et al., Mutat. Res. 275:395-403, 1992; Dunn, J. Cell Biol. 110:1923-1933, 1990; Cataldo and Nixon, Trends Neurosci. 18:489-496, 1995; Seglen et al., Semin. Cell Biol. 1:441-448, 1990). Moreover, regulated autophagy is a key mechanism by which cells control their size (Cataldo and Nixon, Trends Neurosci. 18:489-496, 1995). This function may be particularly relevant to the issue of cell atrophy as a neuropathological feature and antecedent to neurodegeneration in AD (McEwen, Mol. Psychiatry 2:255-262, 1997; Tan et al., J. Neurochem. 71:95-105, 1998). Under conditions of stress, such as amino acid deprivation in hepatocytes, autophagy may account for three-quarters of the cell's protein degradation (Mortimore and

Schworer, Nature 270:174-176, 1977; Seglen et al., Semin. Cell Biol. 1:441-448, 1990). In this situation, autophagy leads to rapid degradation of the cytosol, loss of cellular volume, and cell death within 24 hours (Schwarze and Seglen, Exp. Cell Res. 157:15-28, 1985; Seglen et al., Toxicol. Pathol. 14:342-348, 1986). In this regard, an uncommon "autophagic" neuronal cell death pattern, resembling the pattern in AD, has been described as possibly representing a variant of programmed cell death with a protracted timecourse (Clarke, Anat. Embryol. 181:195-213, 1990; Hornung et al., J. Comp. Neurol. 283:425-437, 1989).

Little has been known about autophagy in neurons. We have discovered, however, that expression of mutant PS1 in cultured cells and in transgenic mice substantially increases autophagy.

Results from the foregoing models also suggest that another pathway to lysosomes, which is non-endosomal and non-autophagic, may be upregulated in PS-FAD. Direct conversion of ER to lysosomes was described by Noda and Farquhar (J. Cell Biol. 119:85-97, 1992) in stimulated thyroid hormone-secreting cells. This little-known pathway is especially interesting because of presenilin's predominant ER localization and the suspected impact of presenilin mutations on the proteolysis of APP within the ER or very close to the ER (Chyung et al., J. Cell Biol. 138:671-680, 1997; Cook et al., Proc. Natl. Acad. Sci. USA 93:9223-9228, 1996; Hartmann, J. Biol. Chem. 272:14505-14508, 1997; Xia et al., Golgi Biochem. 37:16465-16471, 1998).

Changes in the endocytic pathway and lysosomal system in sporadic Alzheimer's disease

Activation of the lysosomal system, evidenced by increased cathepsin gene expression and accumulation of secondary and tertiary lysosomes, is a distinctive response of neurons in sporadic AD (SAD) and DS and an early

marker of metabolic dysfunction in neurons of all vulnerable cell populations in AD (Cataldo et al., Neuron 14:671-680, 1995; Cataldo et al., J. Neuropathol. Exp. Neurol. 55:704-715, 1996; Cataldo et al., Adv. Exp. Med. Biol. 389:271-280, 1996; Cataldo et al., Brain Res. 640:68-80, 1994; Nixon et al., Alzheimer's Disease, in: Advances in Clinical and Basic Research. pp 441-450, 1993). By quantitative image analysis, ~90% of neocortical perikarya in layers III and V of AD brains that demonstrate moderate-severe neuropathology contained 2- to 8-fold elevated numbers of secondary and tertiary hydrolase-positive lysosomes in the absence of chromatolytic or neurofibrillary changes (Cataldo et al., J. Neurosci. 16:186-199, 1996). *In situ* hybridization analysis showed the expression of cathepsin D (Cat D) mRNA to be increased 2-to 3-fold in this cell population in parallel to increases in hydrolase immunoreactivity ($p<0.0001$) (Cataldo et al., Neuron 14:671-680, 1995). Immunoreactivity levels for at least eight different lysosomal hydrolases in neurons were found to be elevated. Changes in the relative proportions of mannose-6-phosphate receptors (MPR) within affected neurons suggest that certain lysosomal hydrolases may also be misdirected to compartments that normally have low hydrolase levels. In SAD, MPR46, but not MPR215 immunoreactivity is elevated, while in PS-FAD both are increased (Cataldo et al., J. Neurosci. 17:6142-6151, 1997; Nixon et al., Sixth International Conference on Alzheimer's Disease. Vol. 19., Amsterdam, pp S136, 1998). Neurons exhibiting overt atrophy or neurofibrillary changes display robust accumulation of hydrolase-positive lysosomes and lipofuscin, which are then released into the extracellular space following cell lysis (Cataldo et al., Brain Res. 640 68-80, 1994; Cataldo and Nixon, Proc. Natl. Acad. Sci. USA, 87:3861-3865, 1990; Cataldo et al., Proc. Natl. Acad. Sci. USA 88:10998-11002, 1991). These lysosomal compartments, containing a full battery of enzymatically competent hydrolases, persist in the extracellular space

specifically in association with deposits of β-amyloid within both senile and diffuse plaques (Cataldo et al., J. Neuropath. Exp. Neurol. 55:704-715, 1996; Cataldo et al., Brain Res. 640:68-80, 1994; Cataldo and Nixon, Proc. Natl. Acad. Sci. USA 87:3861-3865, 1990; Cataldo, et al., Proc. Natl. Acad. Sci. USA. 88:10998-11002, 1991). Cat D content was >3-fold higher in ventricular CSF from AD patients (n=35) than from 26 patients with Huntington's, diffuse Lewy body, or Pick's disease ($p<0.001$), indicating that Cat D release from affected neurons is an active ongoing process (Schwagerl et al., J. Neurochem. 64:443-446, 1995). As cells degenerate, the persistence of hydrolase-laden compartments in the extracellular space is a unique feature of AD. Extracellular accumulations of lysosomal hydrolases have been observed only in brains of patients with AD or other conditions where β-amyloid accumulates.

Accentuation of lysosomal system upregulation without endosomal system alterations in human Alzheimer's disease brains carrying presenilin mutations

We have found that pyramidal neurons in individuals with moderate to severe AD caused by PS1 mutations do not display the early endosomal abnormalities seen in SAD and DS (Fig. 1). That PS mutations do not alter endosomal function is consistent with recent evidence that ApoE genotype does not affect the onset or severity of AD neuropathology associated with PS-FAD (Cruts and Van Broeckhoven, Ann. Med. 30:560-565, 1998), and that PS effects on Aβ overproduction may be exerted in part via an ER-LS pathway rather than via an endocytic pathway (EP). In contrast to the normal-appearing early endosomes in PS-FAD brains, we observed a substantial upregulation in the numbers of lysosomes (Fig. 2). By Cat D and Cat B immunocytochemistry, we found that the LS activation in the PS-FAD brains was greater than in SAD. Neuronal populations that are less vulnerable in AD, such as those in lamina II

and IV of prefrontal cortex, showed marked lysosomal upregulation in PS-FAD but not SAD.

Lysosomal system abnormalities in $PS1_{M146L}$/APPswe transgenic mice

We have found that many of the same characteristics of LS activation that are seen in AD are also seen in transgenic mice expressing $PS1_{M146L}$ and/or APPswe (Duff et al., Nature 383:710-713, 1996). Like human PS-FAD cases, these transgenic mice do not show abnormally large early endosomes. We have shown that mice expressing APPswe, like AD subjects, have increased intracellular accumulation of lysosomal proteases, such as cathepsins D and B, as well as the non-proteolytic enzyme lysosomal acid phosphatase (LAP), when compared to control littermates. This lysosomal upregulation in APPswe transgenic mice was found at five months of age, prior to plaque deposition. $PS1_{M146L}$ mice, which do not develop plaques, also show LS activation, although in a restricted population of neurons that appears to overlap with the highest levels of PS1 transgene expression (Fig. 3). Both the early appearance of lysosomal changes in the APPswe transgenic mice and the appearance of lysosomal changes in the $PS1_{M146L}$ mice indicate that LS upregulation may occur without β-amyloid deposition. Introduction of both transgenes potentiates the LS abnormalities and hastens and exacerbates neuropathology (Figs. 3 and 4).

Lysosomal enzymes are associated with plaques in transgenic mice

Lysosomal enzymes are closely associated with β-amyloid-containing senile plaques in human AD brain (Cataldo et al., J. Neuropathol. Exp. Neurol. 55:704-715, 1996; Cataldo et al. Brain Res., 640:68-80, 1994; Cataldo and Nixon, Proc. Natl. Acad. Sci. USA 87:3861-3865, 1990). Similarly, lysosomal enzymes (as detected by Cat D immunoreactivity and LAP activity), including

all mannose 6-phosphate-tagged lysosomal hydrolases (shown in Fig. 4), are associated with plaques in $PS1_{M146L}$/APPswe mice and aged APPswe mice. We have now discovered that plaques in the $PS1_{M146L}$/APPswe mice contain degenerating neurites filled with autophagic vacuoles (Fig. 5). By EM, these neuritic profiles contained abundant lysosomes and autophagic vacuoles (AV) and, by LM, increased immunolabeling for LAMP-1, a major lysosome-associated membrane protein.

Increased autophagy in familial Alzheimer's disease transgenic mice

Our immuno- and enzyme cytochemistry and morphologic examinations at the EM level of $PS1_{M146L}$/APPswe mice support our hypothesis that increased neuronal hydrolytic activity is intimately linked to the generation of AD-like pathology. Ultrastructural studies show increased numbers of autophagic vacuoles in the $PS1_{M146L}$/APPswe mice vs. littermate controls and in the $PS1_{M146L}$ mice vs. controls using morphologic criteria combined with LAP activity as a marker for hydrolytic compartments (Fig. 5). AVs are restrictively defined as membrane-enclosed structures in which cellular organelles or cellular contents (such as ribosomes) are identifiable (Cataldo and Nixon, Trends Neurosci. 18:489-496, 1995). Not only were the number of LAP positive lysosomes and AV increased over that seen in control mice, but in the $PS1_{M146L}$/APPswe mice, LAP activity was seen in the trans-most and medial sacules of the Golgi apparatus and frequently, albeit less often, in the cis-most saccule (compare Fig. 3F with Fig. 3H). This unusual distribution throughout the Golgi is indicative of increased LAP biosynthesis. Taken together, our data show an increase in LAP activity and increased numbers of AV within neurons of the $PS1_{M146L}$/APPswe mice, and support a model of increased lysosomal activity and autophagy via a non-endosomal route in some forms of FAD.

Increased autophagy in cells expressing presenilin-I

Importantly, the foregoing observations of lysosomal system activation and increased autophagy are not restricted to transgenic mice expressing mutant PS1. We generated murine L cell lines stably transfected with cDNAs expressing human wild-type PS1 as well as the $PS1_{P117L}$ mutant (Wisniewski et al., Neuroreport 9:217-221, 1998). Equivalent levels of PS1 expression following induction was confirmed using a human-specific PS1 monoclonal antibody generated in this laboratory. When living cells were labeled with monodansyl cadavcine (MDC), a greater number of MDC-positive vacuoles were seen in the L cells expressing the mutant PS1 than in L cells overexpressing wild-type PS1 (Fig. 6) or in non-transfected control cells. MDC is a specific *in vivo* marker for autophagic vacuoles (Biederbick et al., Eur. J. Cell Biol. 66:3-14, 1995). The increased numbers of MDC-positive, autophagic vacuoles seen in L cells expressing mutant PS1 is consistent with our conclusion that autophagy is generally increased by expression of mutant PS1 and that a disruption of autophagy is likely to play a role in the pathology of AD.

We have corroborated these observations in a neuronal-like cell, murine N2a cells (Fig. 7). Autophagic vacuoles were identified by EM ultrastructural morphology in N2a cells overexpressing wild-type PS1, in untransfected cells, and in cells expressing the exon 9 deletion of PS1 (PS1Δ9) (Perez-Tur et al., Neuroreport 7:297-301, 1995). In Fig. 8, quantitative results obtained from counting autophagic vaculoes in 20 randomly chosen fields are displayed. While overexpression of wild-type PS1 showed a small, but statistically significant ($p \leq 0.13$), decrease in the mean number of autophagic vacuoles per field, expression of PS1Δ9 increased the number of autophagic vacuoles per filed over both untransfected N2a cells ($p \leq 0.04$) and over wild-type PS1 overexpressing N2a cells ($p \leq 0.004$). Again, the increased numbers of

autophagic vacuoles seen in the N2a cells expressing the exon 9 deletion-mutant of PS1 further substantiates our observation that autophagy is increased in neurons by expression of mutant PS1.

Lysosomal system abnormalities in cells expressing mutant presenilin-1

In studies using primary cultures of human skin fibroblasts from PS-FAD kindreds, we detected no alterations in early endosomal compartments, but observed an increase in the number of Cat D-positive compartments, comparable to our findings in PS-FAD cases (Fig. 9). Consistent with the possibility of a second, non-endosomal route to the lysosome, we found a number of enlarged vacuolar structures that were immunopositive for the ER-marker protein disulfide isomerase (PDI). The morphology of the PDI-positive compartments was similar to a subpopulation of large LAMP-2 (a lysosomal membrane protein) positive compartments in these same cells (Fig. 9).

One clue to why lysosomal activation may be enhanced by PS mutations is the predominant ER localization of PS. The important established role of the ER in autophagy (Holtzman, E., Plenum Press: Lysosomes, 1989), and the existence of an understudied degradative pathway from the ER and/or Golgi apparatus to the lysosome (Chen et al., J. Cell Biol. 107:2149-2161, 1988; Noda and Farquhar, J. Cell Biol. 119:85-97, 1992), raise the possibility that PS mutations might increase autophagy and/or traffic through this alternative pathway--two scenarios that are supported by our findings.

The discovery of increased activity of pathways from the endoplasmic reticulum to lysosomes allows for the identification of compounds that are useful for the treatment of AD by assessing their ability to decrease activity of pathways from the endoplasmic reticulum to lysosomes.

We can test compounds for their ability to decrease the pathways from the ER to lysosomes in multiple model systems, including but not limited to: (i)

cells in culture; (ii) primary neurons grown in culture; (iii) primary fibroblast lines derived from individuals with PS1 mutations; (iv) cells expressing mutant PS1 or PS2; (v) brains of normal mice; and (vi) brains of transgenic mice. Suitable human cultured fibroblast lines can be derived by antemortem skin biopsy from individuals carrying PS1 mutations (e.g., M233T, S169L, A246G, or M146L), as well as family-member control individuals. Example transfected murine cell lines include the previously described L cells (Kit et al., J. Virol. 1:238-240, 1967) expressing either wild-type human PS1 or the $PS1_{P117L}$ mutant (Wisniewski et al., Neuroreport 9:217-221, 1998) as well as the N2a cells. Other cell lines carrying mutant PS1 or PS2 alleles (e.g., M146L, M146V) are also suitable cell lines for the assays described herein. Alternatively, control and transgenic mice can be used to test the ability of a candidate compound to decrease activity of pathways from the endoplasmic reticulum to lysosomes in an animal model.

Measurements of endoplasmic reticulum to lysosome activity

Autophagy in the foregoing cell lines and animals can be assayed using any of a number of techniques, including the use of monodansyl-cadavarine (MDC), a fluorescent compound that specifically labels autophagic vacuoles (AV) but not early and late endosomes (Biederbick et al., Eur. J. Cell Biol., 66:3-14, 1995). EM morphometric analysis, as well as LM and EM immuno- and enzyme cytochemistry can also be used. We have previously defined AVs by EM as membrane-enclosed structures in which cellular organelles or cellular contents are identifiable (Cataldo and Nixon, Ann. N.Y. Acad. Sci. 679:87-109, 1993; Cataldo and Nixon, Trends Neurosci. 18:489-496, 1995). This definition excludes overlap with other membrane-delimited organelles of the secretory and endocytic pathways. The number and/or size of AVs will be quantified through morphometric analysis with standard statistical computations. The

total area of the cell or region of interest is measured using a standardized grid, and the number of AVs counted and expressed per unit area. The ability of a candidate compound to reduce ER to lysosome conversion in the human or mouse cell lines can also be assayed by double immunofluorescence labeling experiments (Cataldo et al., Brain Res. 640:68-80, 1994) in which coincidence of ER markers and lysosomal markers will be used in cultured cells and in transgenic mouse brain. Coincidence of labeling can be determined using confocal microscopy. Antibodies to resident ER proteins that are useful in these assays include, but are not limited to, protein disulfide isomerase (PDI; StressGen, La Jolla, CA), calnexin (Hochstenbach, Hum. Cell 5:12-24, 1992.), and BiP (StressGen) (Doms et al., Virology 193:545-562, 1993; Hammond and Helenius, Science 266:456-458, 1994). The lysosomal system is labeled using LAP enzyme cytochemistry or antibodies against, for example, Cat D, LAMP-1, or LAMP-2, the latter two being major glycoproteins of the lysosomal membrane (August and Hughes, J. Biol. Chem. 257:3970-3977, 1982; Mathews, J. Cell Biol. 118: 1027-1040, 1992). ER to lysosome conversion can be further assayed by coupling EM morphology with EM immuno- and enzyme cytochemistry, the lumenal protein labeled by immunocytochemistry with, for example, DAB and osmium or by enzyme cytochemistry with lead precipitate, and the membrane protein labeled by immuno-gold. The following pairs of lumenal and membrane markers are provided as examples that are suitable for examination: LAP and calnexin; PDI and LAP; and Cat D and calnexin. Quantitation of compartments showing co-labeling for ER and lysosomal markers can be done as described above for EM morphometric analysis.

A recently identified rab-GTPase, rab24, may play a role in regulating ER/cis-Golgi transition to autophagic vacuoles. Epitope-tagged rab24 has been localized to ER-derived autophagic vacuoles (Barbosa et al., Genomics 30:439-444, 1995; Olkkonen et al., J. Cell Sci. 106:1249-1261, 1993). We have

isolated a cDNA encoding human rab24 and inserted this in-frame into a bacterial GST-fusion protein vector (pGEX-4T; Pharmacia, Piscataway, NJ), which has been expressed in bacteria. To produce antibodies to rab24, we immunize rabbits and test serum for immunoreactivity against GST, GST-rab24, rab24 released from the GST by thrombin digestion, and GST-rab5. Affinity purified antibodies can be characterized using a well established *in vitro* system where amino acid starvation of hepatocytes induces a massive upregulation of cellular autophagy (Seglen et al., J. Cell Biol. 99:435-444, 1984; Seglen et al., Semin. Cell Biol. 1:441-448, 1990). The coincidence of rab24 immunoreactivity and morphologic and immunological indices of autophagy establish the specificity of the rab24 antibody. Using these antibodies, rab24 expression can be used in the assay of drugs in transgenic mice or human or mouse as described above.

Autophagy inhibitors reduce Aβ production and increase tau expression in murine neuroblastoma cells

Based on our finding that increased activity of pathways from the ER to lysosomes is increased in AD, we hypothesize that compounds that decrease this activity are very likely to decrease Aβ formation. We tested this directly by incubating cultured cells in the presence or absence of two different autophagy inhibitors: 3MA or leucine and histidine (Seglen and Gordon, Proc. Natl. Acad. Sci. USA 79:1889-1892, 1982; Seglen and Gordon, J. Cell Biol. 99:435-444, 1984).

Murine neuroblastoma N2a cells expressing the Swedish mutant of APP and wild-type PS1 (APPswe/PS1wt) and N2a cells expressing APPswe/PS1Δ9 (i.e., APPswe and PS1 in which exon 9 has been deleted (Δ9)) were grown in the absence or presence of 3MA (10 mM) or leucine and histidine (10 mM each). Cells were then metabolically labeled for four hours, again in the

absence or presence of autophagy inhibitors. The growth medium was collected and subjected to immunoprecipitation with an anti-Aβ monoclonal antibody that recognizes both Aβ1-40 and Aβ1-42. Labeled Aβ was resolved by SDS-PAGE and fluorography (Fig. 10A). N2a cells produce little endogenous Aβ. Expression of human APPswe greatly increased the amount of Aβ secreted. Inhibiting autophagy with either autophagy inhibitor reduced the amount of Aβ secreted in APPswe/PS1wt expressing cells as well as in the APPswe/PS1Δ9 expressing cells (Fig. 10A), top panel, but did not affect the amount of full-length APP immunoprecipitated from cell lysates with C-terminal antibody (Fig. 10A, bottom panel).

In a second set of experiments, N2a cells expressing APPswe/PS1wt or APPswe/PS1Δ9 were incubated either in the presence or absence of 5mM 3MA (Fig. 10B). Conditioned medium was collected and pooled from two plates and spun at 1000xg to remove cells and cellular debris. The amount of secreted Aβ was then determined using ELISA. In both APPswe/PS1wt and APPswe/PS1 Δ9 N2A cells incubated in the presence of 3MA, there was a reduction of Aβ1-40 and Aβ1-42 in the conditioned medium (Fig. 10B). While the decrease was greatest in the N2A cells expressing mutant PS1, we found a significant decrease of approximately 20% or great for both Aβ species in both the wild-type and Δ9 PS1 expressing lines.

Additionally, we have found that inhibiting autophagy with 3MA or preventing fusion of nascent autophagic vacuoles with lysosomes by vinblastine treatment (50 μM; Kopitz et al., J. Cell Biol. 111:941-953) increased tau protein levels in N2a cells expressing APPswe/PS1Δ9 (Fig. 10C). Tau protein levels were increased by approximately 2 times in 3MA-treated N2a cells, and 2.5 times in vinblastine-treated cells. We have also shown an approximate doubling in tau levels following treatment with leucine and histidine (10 mM each).

Several studies have recently raised the possibility that abnormal forms of tau is metabolized in lysosomes, which is consistent with our hypothesis that organelle turnover by autophagy might involve tau-containing structures. There are numerous assays for tau known in the art. For example, the processing of tau by immunocytochemistry and Western blot analysis is performed using an antibody that recognizes both phosphorylated and non-phosphorylated forms (Adamec et al., Brain Res. 757:93-101, 1997). Abnormal tau conformation and hyperphosphorylated tau is examined using suitable monoclonal antibodies (e.g., MC1 (Jicha et al., J. Neurosci. Res. 48:128-132) and AT8 (Innogenetics, Gent, Belgium)). EM analysis can be performed to characterize cytoskeletal changes in neurons (Nixon, Bioessays 20:798-807, 1998).

We have confirmed this effect of the autophagy inhibitor 3MA on Aβ production in primary cortical neuronal cultures derived from normal mouse embryos (Fig. 11). Following a media change, cultures were incubated in the presence or absence of 3MA for six hours. This media was collected and the levels of Aβ40 and Aβ42 determine by ELISA. 3MA treatment reduced the amount of both Aβ species secreted by the neurons into the media by approximately 20%. This finding indicates that affecting autophagy may have therapeutic value in all forms of AD, not just those resulting from mutations within the presenilin or APP genes. These data indicate that autophagy is a major pathway for the generation of Aβ and that inhibitors of the autophagy pathway, such as 3MA, leucine and histidine, or vinblastine, may significantly reduce the amount of Aβ secreted from neurons, while increasing tau protein levels.

Early diagnosis of Alzheimer's disease

The invention described herein also allows for early diagnosis of AD by the assessment of activity of pathways from the ER to lysosomes in a sample from a patient, compared to a person who does not have AD. The assays described herein are all applicable for this method of diagnosis. The sample can be a cell biopsy, or it can be a biological fluid such as CSF or blood.

Test compounds and extracts

In general, compounds are identified from large libraries of both natural product extracts and synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if

desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-pathogenic activity should be employed whenever possible.

When a crude extract is found to reduce activity of pathways from the endoplasmic reticulum to lysosomes, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having the desired activity. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of lysosomal abnormalities are chemically modified according to methods known in the art.

Uses

For therapeutic uses, the compounds, compositions, or agents identified using the methods disclosed herein may be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Treatment may be accomplished directly, e.g., by treating the animal with antagonists which disrupt, suppress, attenuate, or neutralize the biological events associated with AD. Preferable routes of administration include, for example, inhalation or subcutaneous, intravenous, interperitoneally, intramuscular, or intradermal injections which provide continuous, sustained

levels of the drug in the patient. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of an agent in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's *Pharmaceutical Sciences* by E.W. Martin. The amount of the compound to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease and extensiveness of the disease. A compound is administered at a dosage that decreases activity of the pathways from the endoplasmic reticulum to lysosomes. For example, for systemic administration a compound is administered typically in the range of 0.1 ng - 10 g/kg body weight.

Methods

Primary neuronal cell culture

Dissected embryonic cortical tissue from Sprague-Dawley rat embryos (18d gestation) or mouse embryos (17d gestation) is rinsed with calcium/magnesium-free Hanks buffered saline solution and resuspended in 5.0 ml 0.125% trypsin and 0.5 mM EDTA at 37°C. The suspension is triturated by gentle pipeting and incubated in a shaking water bath for 10 min at 37°C. Trypsin is quenched and the cells are plated at 1.5 x10^6 cells/mL on polylysine coated plates and cultured at 37°C in 5% CO_2 in B27/Neurobasal medium (Gibco/BRL, Gaithersburg, MD) containing 0.0015% fluorodeoxyuridine (FUDR), giving cultures that are >95% neuronal.

Immunocytochemistry and digital confocal microscopic analysis

Sections of aldehyde-fixed human or transgenic mouse brain, or cultured cells are reacted as previously described (Cataldo et al., Neuron 14:671-680, 1995; Cataldo et al., J. Neuropathol. Exp. Neurol 55:704-715, 1996; Cataldo, et

al., Adv. Exp. Med. Biol., 389:271-280, 1996; Cataldo et al., J. Neurosci. 16:186-199, 1996; Cataldo et al., Brain Res. 513:181-192, 1990; Nixon et al., Ann N.Y. Acad. Sci. 679:87-109, 1993; Nixon and Shea, Cell Motil. Cytoskeleton 22:81-91,1992).

Enzyme cytochemistry

For demonstration of acid phosphatase activity, 0.0015M cytidine-3í,5í-cyclic monophosphate (Sigma) in 0.1 M tris-maleate buffer, pH 5.5, is used as substrate (A. Novikoff, Lysosomes in the physiology and pathology of cells. Little Brown and Co., Boston, pp. 36, 1963; A. Novikoff, Lysosomes in nerve cells. Elsevier Publishing Co., N.Y., pp. 319, 1967) for glucose-6-phosphatase activity, 0.00175 M glucose-6-phosphate (Sigma Chemicals, St. Louis, MO) in 0.1 M Tris-maleate buffer, pH 6.8, is used (Broadwell et al., J. Histochem. Cytochem. 31:1077-1088, 1983; Broadwell et al., J. Histochem. Cytochem. 31:818-822, 1983). Incubation proceeds at 37° C for 30-90 min. Enzymatically released phosphate is trapped as a lead precipitate visualized directly in electron microscopy and after conversion to a lead sulfide in light microscopy.

In the brains of transgenic mice overexpressing APPswe, PS1wt, $PS1_{M146L}$ (or combinations thereof) in the presence and absence of candidate compounds, we assess the LS using sMPR (Valenzano et al., Anal. Biochem. 209:156-162, 1993); antibodies specific for Cat D and cystatin C; an *in situ* enzyme assay for LAP (Novikoff, Elsevier Publishing Co., N.Y., pp. 319, 1967); and antibodies specific for MPR46 and MPR215 (Cataldo et al., J. Neurosci. 17:6142-6151, 1997). Tissue sections from the selected anatomical regions are processed for immuno- and enzyme-cytochemistry as previously described (Cataldo et al., J. Neuropathol. Exp. Neurol. 55:704-715, 1996; Cataldo et al., Adv. Exp. Med. Biol. 389:271-280, 1996; Cataldo et al., Brain

Res. 640:68-80, 1994). In addition to immunochemistry, these markers are used in Western blot analysis. Additional EP and LS markers known to those in the art can also be useful in the present method.

LAP activity in brain homogenates is determined using a colorimetric assay based on the hydrolysis of 10 mM p-nitrophenyl phosphate in 0.1 M sodium citrate, pH 4.5 (Saftig et al., J. Biol. Chem. 272:18628-18635, 1997). Aβ levels and β-amyloid burden are determined by a quantitative ELISA assay and by image analysis of β-amyloid plaque density, respectively. Indices of LS upregulation include an increase in lysosome numbers and density of lysosome-specific labels in neurons; increases in lysosomal enzyme activity and/or the levels of lysosomal hydrolases detected by Western blot analysis; and changes in lysosomal enzyme distribution, including the presence of lysosomal hydrolases in the secretory pathway, in endocytic compartments, and associated extracellularly with plaque. We can examine proteolytic (e.g. Cat D), and non-proteolytic (LAP) lysosomal hydrolases, and the proteins responsible for targeting most acid hydrolases to the lysosome (MPR215, MPR46), and the endogenous inhibitor cystatin C.

Isolation and assay of cathepsins

Cat D is assayed as pepstatin-inhibitable activity in the TCA soluble fraction prepared from brain homogenate according to Nixon & Marotta (Marotta, C.A. and Nixon, R.A., J. Neurochem, 43: 507-516, 1984), using ^{14}C-labelled methemoglobin. Cat B and Cat L are assayed according to the protocol of Barret and Kirschke (Barrett, A.J. and Kirschke, H., Methods Enzymol, 80: 535-561, 1981) by measuring amc released from Z-Arg-Arg-amc (specific for Cat B) and Z-Phe-Arg-amc (specific for Cat B and L). Immunoreactive Cat B and D are measured by Western blot analysis as described (Mohan and Nixon, J. Neurochem. 64:859-866, 1995) using

polyclonal antibodies raised against the mature forms of Cat D and Cat B as probes.

Aβ ELISA methods

The Aβ sandwich ELISA is generally known to those skilled in the art, with both Aβ ELISA kits (Biosource International, Camaville, CA) and appropriate antibodies (e.g., 4G8, 6E10; Saneteck PLC, Napa, CA) commercially avaliable.

For the Aβ sandwich ELISA, Nunc-Immuno Plates (Nunc A/S, Roskilde, Denmark) were coated overnight using 4^{0}C using antibodies specific for Aβ40 or Aβ42 in 100 mM bicarbonate buffer, pH 9.6. Remaining protein binding sites were blocked by incubating with 1% Block Ace (Yukijirushi Milk, Sapporo Japan) in PBS for 4 hours at room temperature. 10% (w/v) homogenates were prepared from a hemibrain in 20MM Tris, 250 mM sucrose, 1mM EDTA, 1mM EGTA, protease inhibitors, pH 7.4, and stored frozen at -70^{0}C. Immediately prior to being loaded on the ELISA, 1ml of the brain homogenate was extracted in diethylamine (Sigma, St. Lousi, MI) by adding an equal volume of 0.4% DEA in 100 mM NaCl, re-homogenized and centrifuged for 1 hour at 100,000 x g. The supernatant was collected, neutralized with 0.1 volume 0.5 M Tris, pH 6.8, and loaded in duplicate wells both neat and diluted 1:2 in EC buffer (20mM Na phosphate, 2mM EDTA, 400 mM NaC1, 0.2% BSA, 0.4% Block Ace, 0.95% CHAPS). This DEA extraction protocol has been shown to efficiently recover immunoreactive Aβ from mouse brain homogenates and leave both full-length and sAPP in the 100,000 xg pellet (Savage et al., 1998). Alternatively, conditioned media collected from cells was loaded neat and 1:2. Aβ-40 and Aβ-42 peptide standards were purchased from American Peptide Co. (Sunnyvale, CA), stored at -70^{0}C, and diluted in EC buffer immediately prior to use. ELISA plates were incubated overnight

with 4^0C with samples and standards. Aβ was detected by incubating for 4 hours at room temperature with an HRP-conjugated anti-Aβ antibody in 20mM Na phosphate, 2mM EDTA, 400 mM Nacl, 1.0% BSA. ELISA plates were developed using a color reaction (ABTS Peroxide Substrate System, Kirkegaard & Perry, Gaithersburg, MD) and the OD_{450} read.

Additional methods

A compound that decreases the activity of pathways from the ER to lysosomes can be further tested for AD-like abnormalities in physiology, anatomy, or behavior using assays known to those skilled in the art, including those described in U.S. patent 5,877,399, hereby incorporated by reference.

Other Embodiments

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations following, in general, the principles of the invention and including such departures from the present disclosure within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is:

1. A method of diagnosing probable neuronal atrophy-associated dementia in a human patient, said method comprising the steps of:

(a) determining the level of activity of pathways from the endoplasmic reticulum to lysosomes in said patient; and

(b) comparing the level of said activity to normal levels, wherein an increase in the level of said activity, relative to normal levels, indicates neuronal atrophy-associated dementia.

2. The method of claim 1, wherein step (a) comprises measuring the level of said activity of pathways from the endoplasmic reticulum to lysosomes in a cell of said patient.

3. The method of claim 2, wherein said cell is selected from the group consisting of a neuron, a fibroblast, and an endothelial cell.

4. The method of claim 1, wherein step (a) comprises measuring the level of said activity of pathways from the endoplasmic reticulum to lysosomes in a biological fluid of said patient.

5. The method of claim 1, wherein said neuronal atrophy-associated dementia is Alzheimer's disease.

6. A method for identifying a candidate compound as a compound that is useful for the treatment of neuronal atrophy-associated dementia, said method comprising the steps of:

(a) providing a cell;

(b) contacting said cell with a candidate compound; and

(c) determining the activity of pathways from the endoplasmic reticulum to lysosomes, wherein a decrease in said activity, relative to the activity in a control cell not contacted with said candidate compound, identifies the candidate compound as a compound that is useful for the treatment of neuronal atrophy-associated dementia.

7. The method of claim 6, wherein said cell is in an animal selected from the group consisting of a mouse, rat, dog, cat, monkey, and human.

8. The method of claim 6, wherein said cell is from a human or a mouse.

9. The method of claim 6, wherein said cell comprises a polypeptide comprising a mutation that is present in a human with familial Alzheimer's disease.

10. The method of claim 9, wherein said polypeptide is presenilin-1 or presenilin-2.

11. The method of claim 10, wherein said mutation is P117L, M146L, M146V, S169L, M233T, or A246G.

12. The method of claim 9, wherein said polypeptide is APP.

13. The method of claim 6, wherein said cell is *in vitro*.

14. The method of claim 6, wherein said cell is selected from the group consisting of a fibroblast, an endothelial cell, and a neuron.

15. The method of claim 6, wherein said neuronal atrophy-associated dementia is Alzheimer's disease.

16. A method for identifying a candidate compound as a compound that is useful for the treatment of neuronal atrophy-associated dementia, said method comprising the steps of:

(a) providing a cell;

(b) contacting said cell with a candidate compound that decreases the activity of pathways from the endoplasmic reticulum to lysosomes; and

(c) determining the ability of the cell to withstand a cytotoxic challenge selected from the group consisting of oxidative stress, Aβ, hypoxia, and metabolic challenge, wherein an increase in survival of said cell, relative to survival of a cell not contacted with said compound, identifies said compound as a compound that is useful for the treatment of neuronal atrophy-associated dementia.

17. The method of claim 16, wherein said cell is in an animal selected from the group consisting of a mouse, rat, dog, cat, monkey, and human.

18. The method of claim 16, wherein said cell is from a human or a mouse.

19. The method of claim 16, wherein said cell comprises a polypeptide comprising a mutation that is present in a human with familial Alzheimer's disease.

20. The method of claim 19, wherein said polypeptide is presenilin-1 or presenilin-2.

21. The method of claim 20, wherein said mutation is P117L, M146L, M146V, S169L, M233T, or A246G.

22. The method of claim 19, wherein said polypeptide is APP.

23. The method of claim 16, wherein said cell is *in vitro.*

24. The method of claim 16, wherein said cell is selected from the group consisting of a fibroblast, an endothelial cell, and a neuron.

25. The method of claim 16, wherein said neuronal atrophy-associated dementia is Alzheimer's disease.

26. A method for identifying a candidate compound as a compound that is useful for the treatment of neuronal atrophy-associated dementia, said method comprising the steps of:

(a) providing a cell;

(b) contacting said cell with a candidate compound that decreases the activity of pathways from the endoplasmic reticulum to lysosomes; and

(c) determining the levels of Aβ produced by the cell, wherein a decrease in the levels of Aβ produced by said cell, relative to a control cell not contacted with said candidate compound, identifies the candidate compound as a compound that is useful for the treatment of neuronal atrophy-associated dementia.

27. The method of claim 26, wherein said cell is in an animal selected from the group consisting of a mouse, rat, dog, cat, monkey, and human.

28. The method of claim 26, wherein said cell is from a human or a mouse.

29. The method of claim 26, wherein said cell comprises a polypeptide comprising a mutation that is present in a human with familial Alzheimer's disease.

30. The method of claim 29, wherein said polypeptide is presenilin-1 or presenilin-2.

31. The method of claim 30, wherein said mutation is P117L, M146L, M146V, S169L, M233T, or A246G.

32. The method of claim 29, wherein said polypeptide is APP.

33. The method of claim 26, wherein said cell is *in vitro*.

34. The method of claim 26, wherein said cell is selected from the group consisting of a fibroblast, an endothelial cell, and a neuron.

35. The method of claim 26, wherein said neuronal atrophy-associated dementia is Alzheimer's disease.

36. A method for treating a patient with neuronal atrophy-associated dementia, said method comprising administering to said patient a compound that decreases the activity of pathways from the endoplasmic reticulum to lysosomes.

37. The method of claim 36, wherein said patient has sporadic Alzheimer's disease, familial Alzheimer's disease, or Down's syndrome.

38. The method of claim 36, wherein said patient has a mutation in their presenilin-1 gene, presenilin-2 gene, or APP gene.

39. The method of claim 36, wherein said compound is selected from the group consisting of 3-methyladenine, a derivative of 3-methyladenine, leucine, histidine, and vinblastine.

40. The method of claim 36, wherein said compound also reduces Aβ formation.

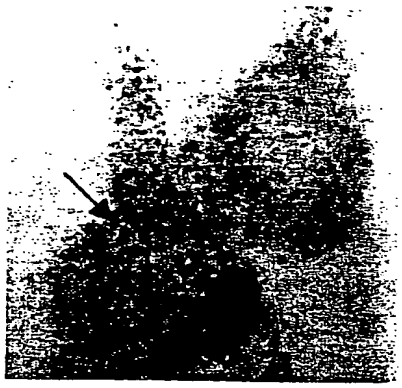


Fig. 1A

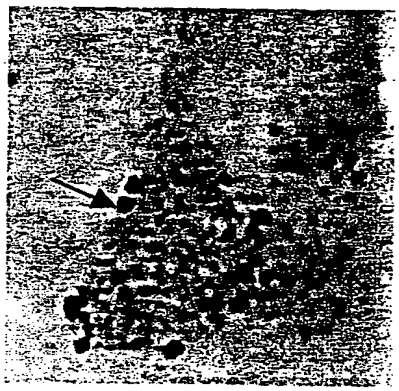


Fig. 1B

Fig. 1C

Fig. 1D

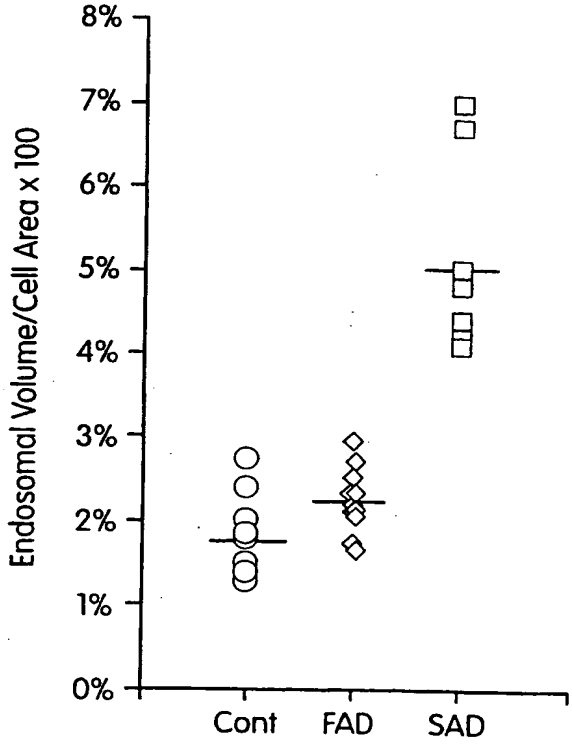


Fig. 1E

Fig. 2C

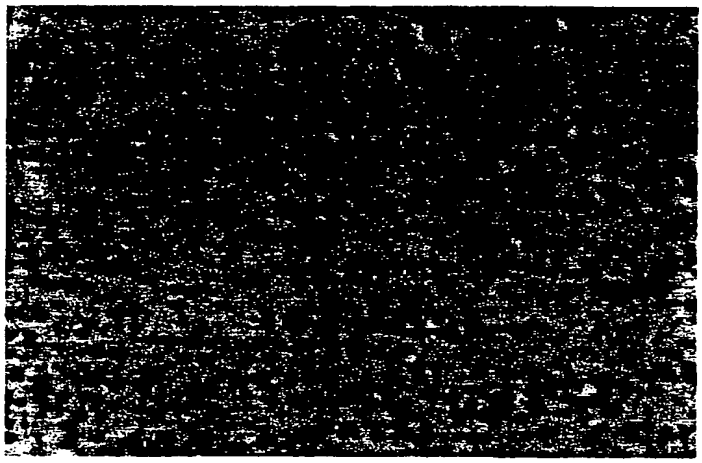


Fig. 2B

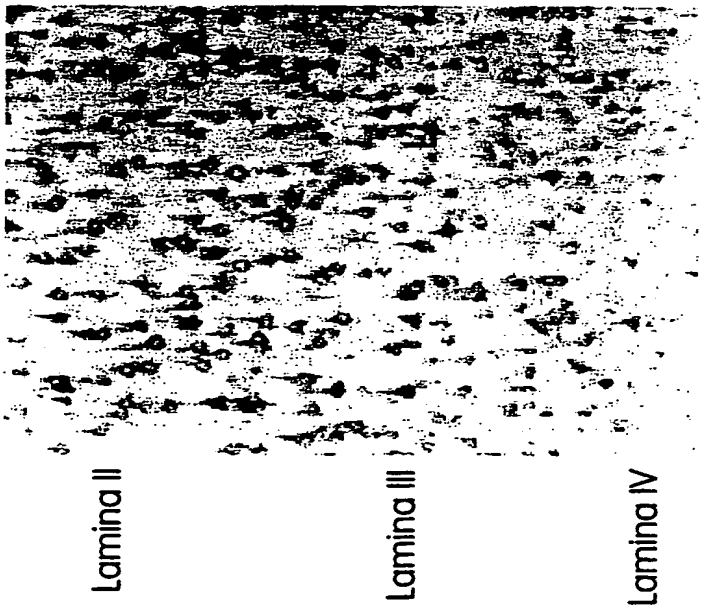


Fig. 2A

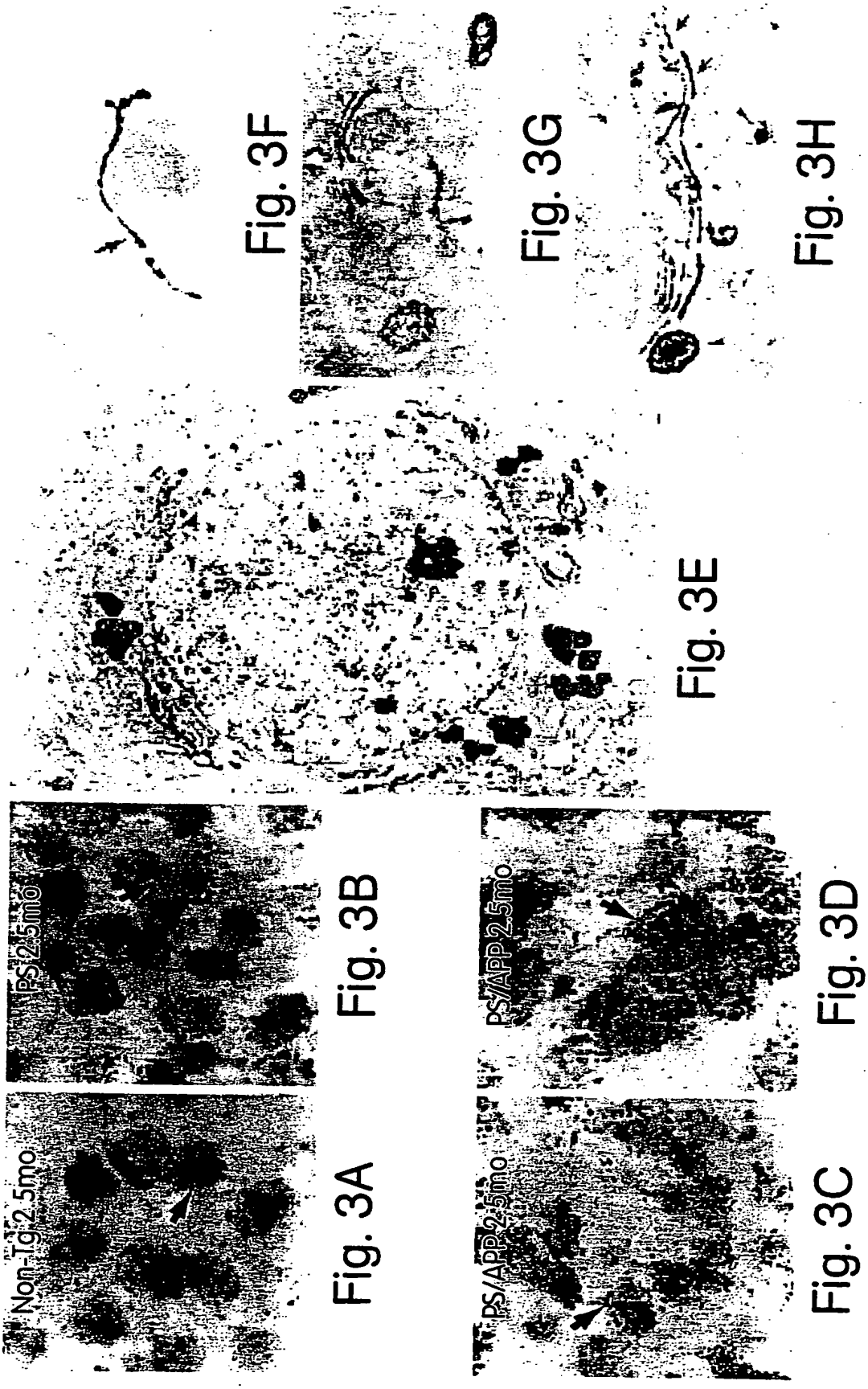


Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D

Fig. 3E

Fig. 3F

Fig. 3G

Fig. 3H

PS/APP 2.5 mo

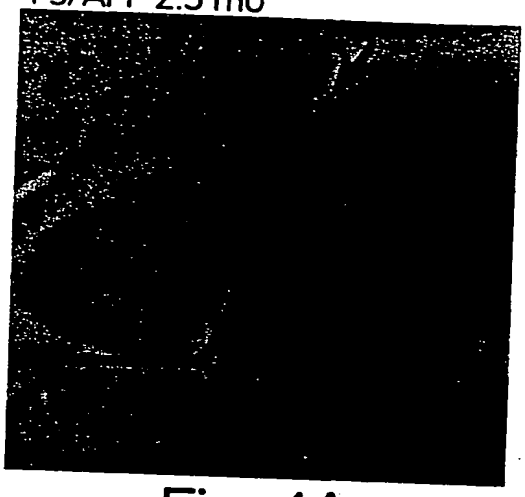


Fig. 4A

PS/APP 5.5 mo

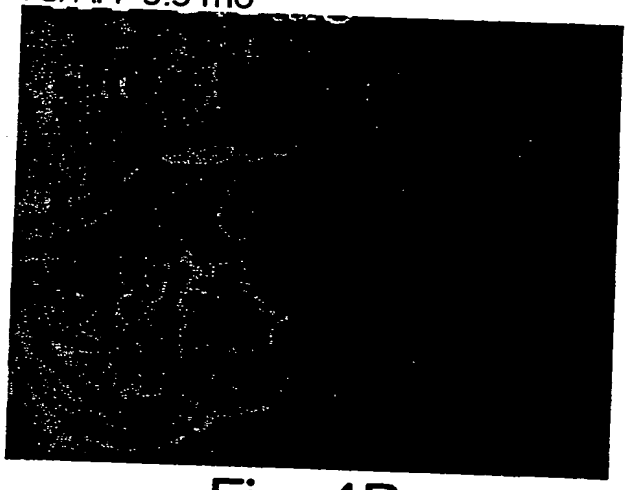


Fig. 4B

PS/APP 9 mo

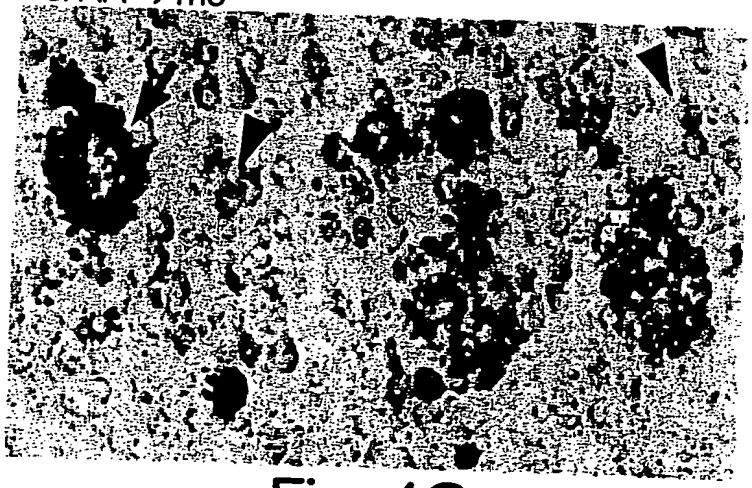
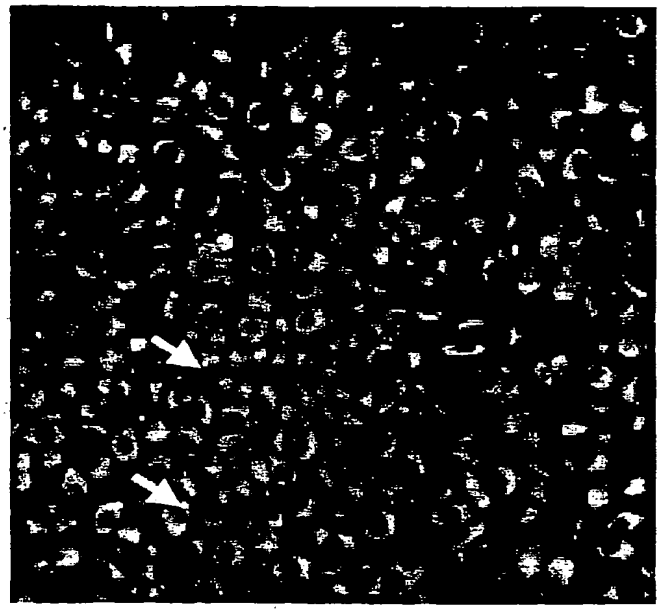


Fig. 4C

Fig. 5A

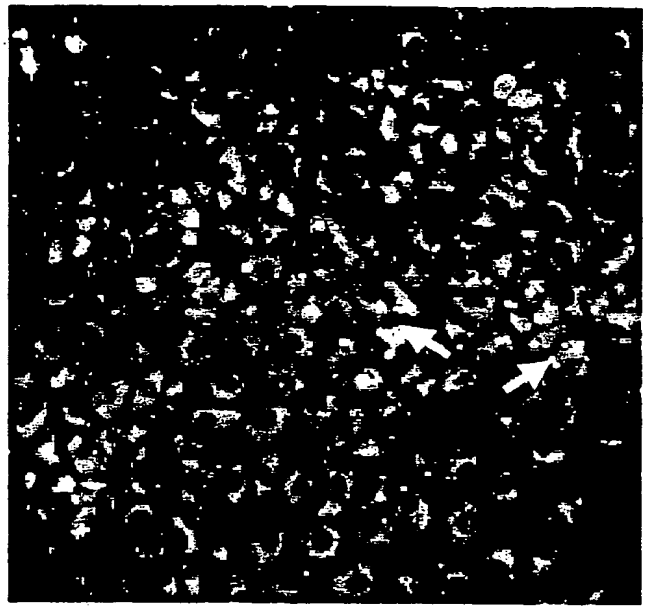
Fig. 5B

Fig. 5C



untransfected

Fig. 6A

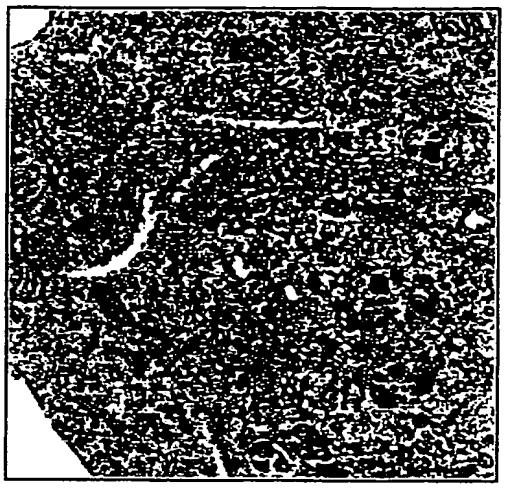


PS1P117L

Fig. 6B

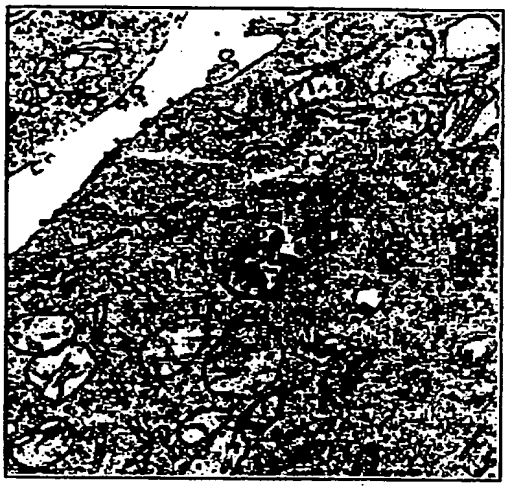
PS1P117L

Fig. 6C



non-transfected

Fig. 7A



PS1wt/APPswe

Fig. 7B

PS1Δ9/APPswe

Fig. 7C

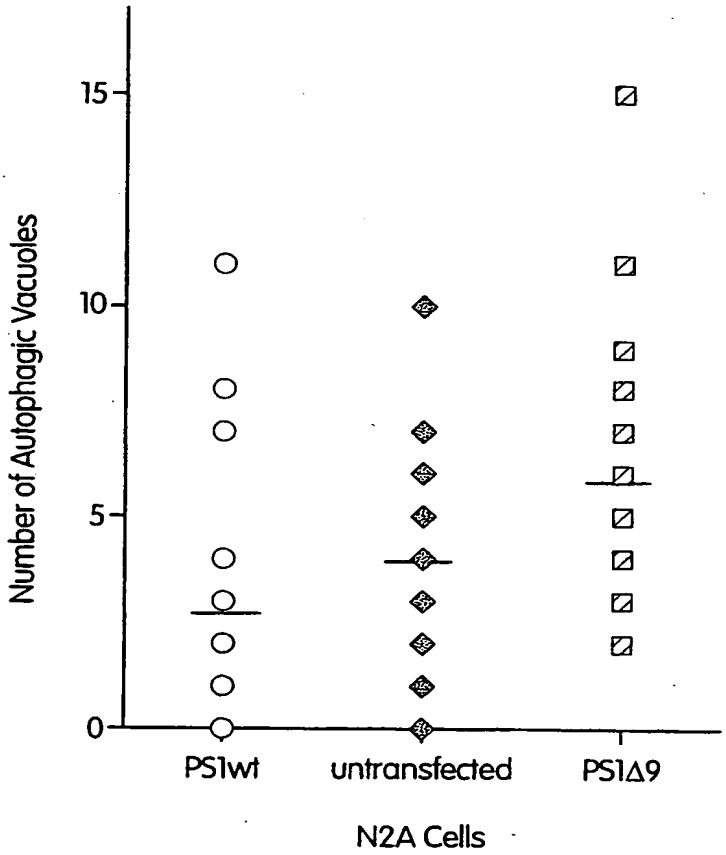


Fig. 8

Fig. 9A

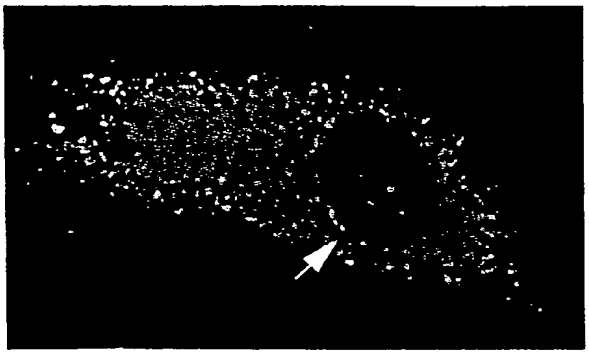


Fig. 9B

Fig. 9C

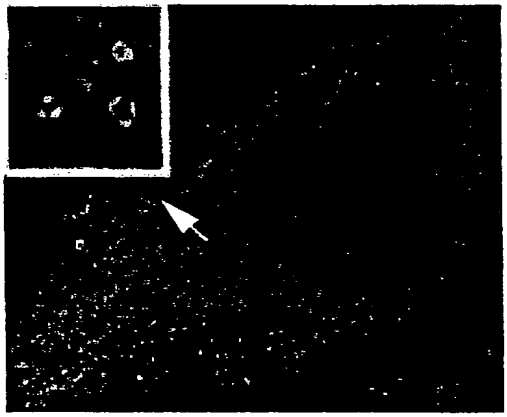


Fig. 9D

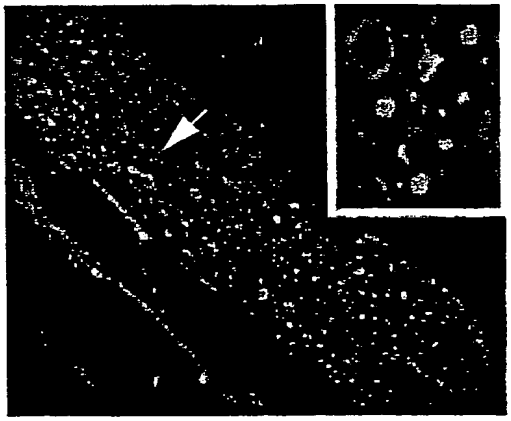


Fig. 9E

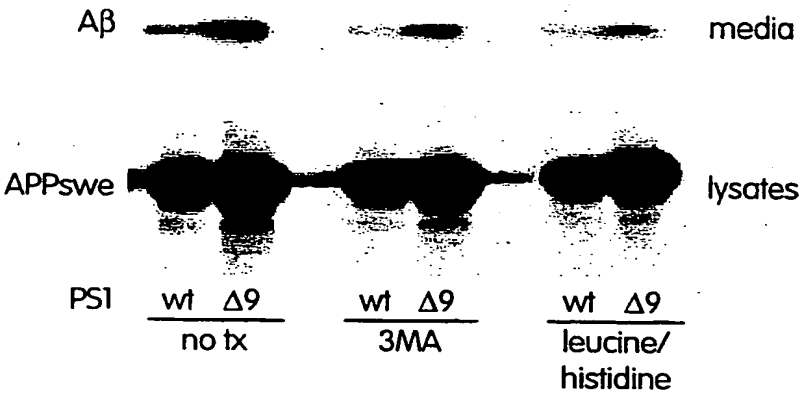


Fig. 10A

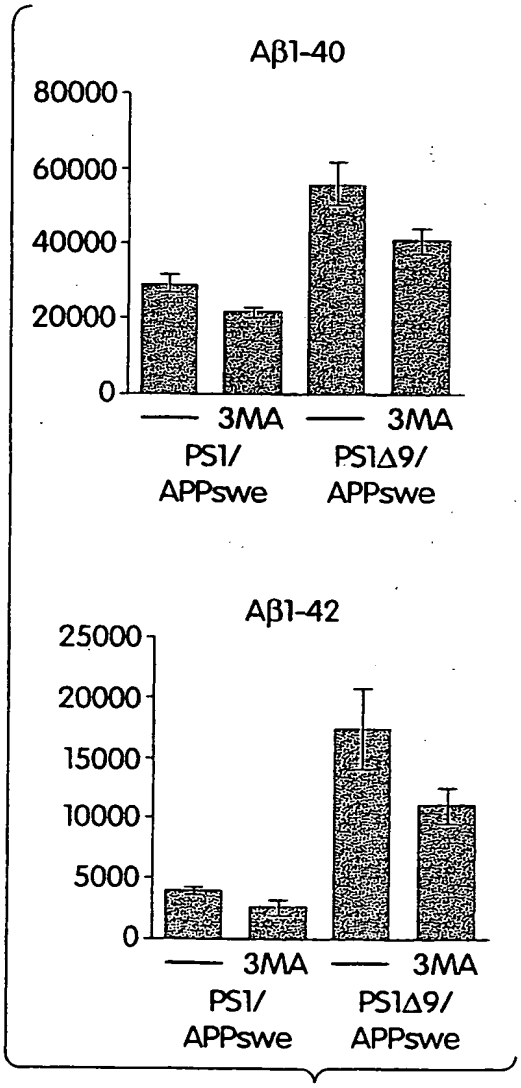


Fig. 10B

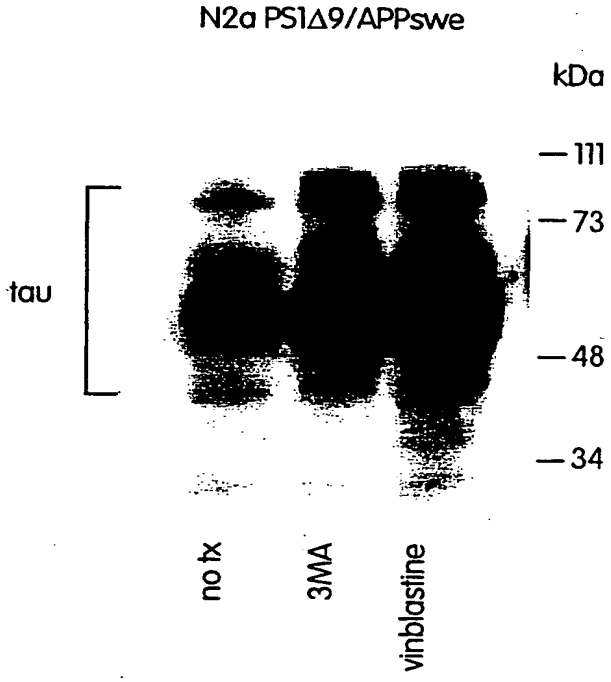


Fig. 10C

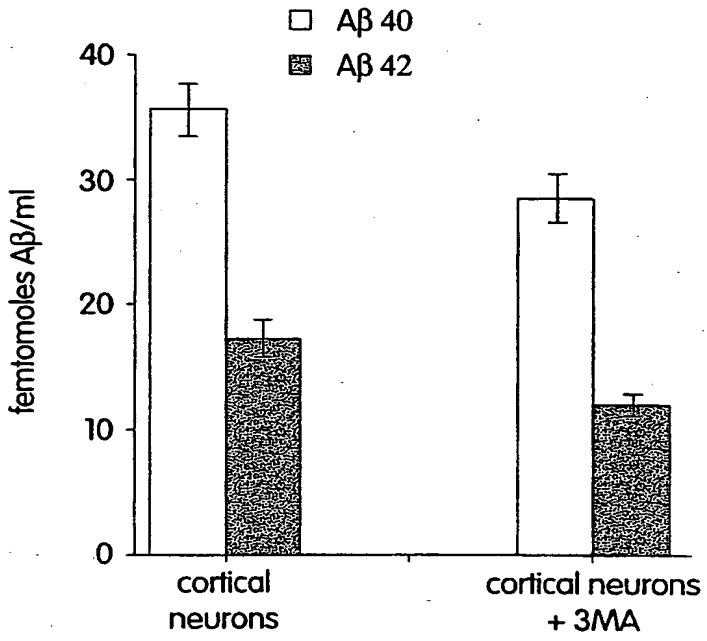


Fig. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11739

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 5/00, 14/00; A61K 38/00, 38/43, 39/395

US CL :530/300, 350; 424/130.1, 178.1, 94.1; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 424/130.1, 178.1, 94.1; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Author search

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, CAPlus, Genbank, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	CATALDO, A.M. et al. Properties of the Endosomal-Lysosomal System in the Human Central Nervous System: Disturbances Mark Most Neurons in Populations at Risk to Degenerate in Alzheimer's Disease. J of Neurosci., 01 January 1996, Vol. 16, No. 1, pages 186-199, see entire document.	1-3, 5 ---- 4, 6-40
X -- Y	BUSCA et al. The Carboxy-Terminal Region of Human Lipoprotein Lipase is Necessary for its Exit From the Endoplasmic Reticulum. J. of Lipid Res. April 1998, Vol. 39, pages 821-833, see entire document.	1-3 ---- 4-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
23 AUGUST 2000	07 SEP 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SHARON L. TURNER
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11739

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	YAN ZHOU et al. A Mutation in a Mild Form of Galactosialidosis Impairs Dimerization of the Protective Protein and Renders it Unstable. EMBO J., 1991, Vol. 10. No. 13., pages 4041-4048, see entire document.	1-3 --- 4-40
X -- Y	IKEGAMI et al. Immunohistochemical Examination of Phosphorylated Tau in Granulovacuolar Degeneration Granules. Psych. & Clin. Neurosci. 1996, Vol. 50, pages 137-140, see entire document.	1-3 --- 4-40
X -- Y	PUNNONEN et al. Effects of Vinblastine, Leucine, and Histidine, and 3-Methyladenine on Autophagy in Ehrlich Ascites Cells. Exper. & Mol. Path., 1990, Vol. 52., pages 87-97, see entire document.	6-35 ---- 1-5, 36-40
X -- Y	CATALDO et al. Lysosomal Abnormalities in Degenerating Neurons Link Neuronal Compromise to Senile Plaque Development in Alzheimer Disease. Brain Research. 1994, Vol. 640, pages 68-80, see entire document.	1-3 -- 4-40
X -- Y	WATARI et al. Niemann-Pick C1 Protein: Obligatory Roles for N-Terminal Domains and Lysosomal Targeting in Cholesterol Mobilization. PNAS. February 1999, Vol. 96, pages 805-810, see entire document	1-3 --- 4-40
X -- Y	DASH et al. Inhibitors of Endocytosis, Endosome Fusion, and Lysosomal Processing Inhibit the Intracellular Proteolysis of the Amyloid precursor protein. Neurosci. Lttrs., 1993, Vol. 164. pages 183-186, see entire document.	6-35 --- 1-5, 36-40
X -- Y	BJARNADOTTIR et al. Intracellular Accumulation of the Amyloidogenic 168Q Variant of Human Cyststin C in NIH/3T3 Cells. J. Clin. Pathol: Mol Pathol. 1998, Vol. 51, pages 317-326, see entire document.	1-3 -- 4-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11739

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11739

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-5, drawn to a method of diagnosis.
Group II, claim(s) 6-15, drawn to a method for identifying a candidate compound.
Group III, claim(s) 16-25, drawn to a second method for identifying a candidate compound.
Group IV, claim(s)26-35, drawn to a third method for identifying a candidate compound.
Group V, claim(s) 36-40, drawn to a method for treating a patient.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The methods differ in steps, technical features, reagents and outcomes. For example the method of group I diagnoses dementia with the special technical feature of activity levels of pathways from the ER to lysosomes. The method of group II identifies candidate agents with the technical feature of decreasing ER to lysosome activity. The method of group III identifies candidate compounds with the technical featue of determinin the ability of the cell to withstand cytotoxic challenge. The method of group IV identifies candidate compounds with the technical feature of decrease levels of A-Beta. The method of group V is a method for treating a patient with a technical feature of decreasing the activity of pathways from the ER to lysosomes in the patient.

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